Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis

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ABSTRACT

Exosomes are naturally occurring biological nanovesicles utilized by tumors to communicate signals to local and remote cells and tissues. Melanoma exosomes can incite a pro-angiogenic signaling program capable of remodeling tissue matrices. In this study, we demonstrate exosome-mediated conditioning of lymph nodes and define microanatomic responses that license metastasis of melanoma cells. Homing of melanoma exosomes to sentinel lymph nodes imposes synchronized molecular signals that effect melanoma cell recruitment, extracellular matrix deposition and vascular proliferation in the lymph nodes. Our findings highlight the pathophysiological role and mechanisms of an exosome mediated process of microanatomical niche preparation that facilitates lymphatic metastasis by cancer cells.
INTRODUCTION

In order to metastasize, tumor cells must manipulate their microenvironment to optimize conditions for deposition and growth both locally and at a distance. In accordance with the “seed and soil” hypothesis for example cancer stem cells or metastatic cells function as “seeds” and a particular organ microenvironment or niche serves as the “soil” (1-3). Potential sites for remote tumor implantation might thus be prepared well ahead of actual metastasis (4).

For specific cancers such a metastatic melanoma, the process of metastasis involves lymphatic dissemination although the precise role lymph nodes play in supporting this process is not defined (5). In one hypothesis melanoma cells undergo simultaneous hematogenous and lymphatic spread and the presence of tumor cells in sentinel or regional nodes is merely indicative of metastasis. Alternatively, sentinel or regional nodes play an active role in the progression of melanoma metastasis. The observation that regional lymph nodes downstream of melanomas undergo reactive lymphangiogenesis prior to metastasis (6) suggest that melanoma metastasis is facilitated by preparation of a pre-metastatic niche within lymph nodes. This process is believed to be mediated by tumor secretion of paracrine angiogenic growth factors.

In this report we demonstrate an adjunctive and highly efficient model of pre-metastatic niche formation in regional lymph nodes through the local actions of melanoma exosomes. Exosomes are naturally occurring biological nanovesicles (~30-100 nm) that are formed by the inward budding of multivesicular bodies (MVBs), as a component of the endocytic pathway (7-11). They are generated constitutively and released into the tumor microenvironment and circulation via fusion of multivesicular bodies with the tumor cell plasma membrane. The nanoscale size of exosomes facilitates their penetration and interaction with local tumor cells as well as with cell types that are distant to an advancing tumor cell front. This may result in tumor immune evasion by direct suppression of T cell activation and induction of apoptosis (12), suppression of the anti-tumor activity of natural killer cells (13) and other mechanisms (14).
Recently, we have shown that melanoma exosomes induce alterations in the angiogenic microenvironment using a 3D culture assay (15). These results suggest that melanoma exosomes may be instrumental in melanoma cell dissemination. These findings support other studies demonstrating increased endothelial tubulogenesis by D6.1A tetraspanin expressing pancreatic cancer cell exosomes (16) or increased migration, proliferation, sprouting and upregulation of vascular endothelial growth factor receptor 1 (VEGFR-1) on endothelial cells by tetraspanin 8 expressing rat adenocarcinoma cell exosomes (17). Moreover, Dll4 (Delta-like 4), a Notch receptor ligand, can be transferred by tumor exosomes to endothelial cells resulting in increased endothelial tubule branching (18).

Thus, in conjunction with the findings of others, our previous observations suggest the presence of a “melanoma exosomal messenger system” that exhibits multifunctional paracrine bioactivities that might facilitate tumor communication within the local tumor microenvironment and distantly through upregulation of angiogenic processes (15). This in vivo investigation explores the hypothesis that melanoma exosomes can condition sentinel lymph nodes to become remote niches conducive to the recruitment and growth of melanoma cells. The experimental strategy entails production and isolation of melanoma exosomes in vitro, followed by preconditioning of nodes with either tumor exosomes versus bland liposomes as a control, and then tumor cell injection and lymphatic tracking to define the molecular signaling events and microanatomic responses that prepare the metastatic turf.

MATERIALS AND METHODS

Materials and cell culture. Mouse B16-F10 (CRL 6475) melanoma cells and media were purchased from ATCC (August 2008, Manassas VA), MAP and mycoplasma tested for purity and kept frozen at -80 °C under liquid nitrogen until resuscitated for use. For culture, cells were maintained with 90% DMEM and 10% heat inactivated fetal bovine serum at 37°C and 5% CO₂.
Male 6-8 week old albino C57/BL6 mice, B6(Cg)-Tyr<sup>−/−</sup>/J, were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on a normal diet until use. Animal care was in accordance with institutional guidelines. Albino mice were used to minimize fluorescent absorption by melanin leading to signal loss. Fluorescent lipophilic tracers DiO (3,3'-dihexadecyloxacarbocyanine Ex. 484 / Em. 501), Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine Ex. 549 / Em. 565), DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine Ex. 644 / Em. 665), and DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethylindotrificarbocyanine Ex. 750 / Em. 780) were purchased from Invitrogen (Carlsbad, CA).

**Isolation and labeling of exosomes.** B16-F10 melanoma exosomes were isolated for use in experiments according to previously established methods (15). Briefly, B16-F10 melanoma cell cultures were grown to 70% confluence in three 300 cm<sup>2</sup> flask. Culture media was removed and cells washed in PBS. Cells were cultured for 48 hrs in the presence of conditioned media. Conditioned culture media was prepared by subjecting normal culture media to overnight ultracentrifugation at 110,000 x g to remove bovine exosomes (19). B16 melanoma exosomes were collected from 48 hr culture in conditioned media through standard differential centrifugation steps using a 70 Ti rotor (19). Culture media was spun and supernatants collected from 300 x g for 10 min, 2000 x g for 10 min, to remove residual cells and debris, 10,000 x g for 30 min to remove microparticles (20) and 100,000 x g for 2 h in the presence or absence of 1.0 μM Dil or DiR. Exosome pellets were washed three times in PBS, pooled, and re-isolated in PBS at 100,000 x g for 2 hr. Exosome pellets were resuspended in 1 ml of PBS, protein content measured via BCA absorbance (Thermo Fisher Scientific Inc., Rockford, IL) and stored at -80°C until use. Between the 10,000 and 100,000 x g centrifugation steps, exosomes were sized using dynamic light scattering (DLS) as previously reported (15) and the electrokinetic potential (zeta potential) of purified exosomes in PBS was measured using a Zeta
Melanoma Exosome Preparation of Lymph Nodes for Metastasis

Plus Zeta Potential Analyzer (Brookhaven Instruments Corp. Holtsville, NY). Previously, fluorescent exosome localization technique (FELT) was used to confirm the use of differential centrifugation and dynamic light scattering in obtaining a purified population of B16-F10 melanoma exosomes from conditioned media (15). Using FELT, B16-F10 melanoma exosomes applied to continuous sucrose gradients (2.0 - 0.25 M sucrose, 20 mM HEPES/NaOH, pH 7.4), were found to have a density of (1.10 – 1.21 g/ml) (15).

**Construction of fluorescent control liposomes.** A lipid commixture including 64.89 mole% lecithin (phosphatidylcholine, Avanti Polar Lipids Inc., Alabaster AL), 32.08 mol% cholesterol (Sigma-Aldrich Co., St. Louis, MO), 3.02 mole% phosphatidylethanolamine (Avanti Polar Lipids Inc., Alabaster, AL), and 0.01 mol% DiD was solubilized in chloroform, and dried to a lipid film under continuous vacuum using a rotary evaporator. Residual solvent was removed by overnight drying under continuous vacuum. The dry lipid film was resuspended in 20 ml of distilled deionized water, and emulsified (Microfluidics Corp., Newton, MA) at 20,000 psi for 4 minutes to form liposomes. Liposomes in PBS were sized using DLS and zeta potential determined using a Zeta Plus Zeta Potential Analyzer (Brookhaven Instruments Corp. Holtsville, NY).

**Nodal trafficking of liposomes, exosomes or cells.** Fluorescent DiR or Dil labeled exosomes (50 μg), DiD-liposomes or DiO-B16-melanoma cells (1 million) were each injected into the foot pads of individual mice using established techniques (21). To equalize the number of liposomes and exosomes injected, a standard curve relating counts of various particle concentrations of liposomes, designed to be approximately the same size (~100 nm) as B16-F10 melanoma exosomes was constructed based on dynamic light scattering (Brookhaven Instruments Corp., Holtsville, NY). A best fit equation with R² ~ 1.0 was then generated and used to mathematically predict the number of exosomes present in a 50 μg sample. Based on this calculation, an equivalent number of DiD-liposomes in PBS (50 μl) to 50 μg of DiR-
exosomes in PBS (50 μl) were used. A similar standard curve was constructed for DiO labeled melanoma cells to convert fluorescent efficiency values as measured using a Xenogen in vivo imaging system (IVIS) Spectrum Workstation (Caliper Life Sciences, Hopkinton, MA) to cell numbers.

**Lymph node dissection and fluorescent microscopy.** Animals were anesthetized with 2.5% isoflurane and euthanized by cervical dislocation under deep anesthesia. The left and right popliteal (PO) or inguinal (IN) murine lymph nodes as mapped by Harrell et al (21) were dissected, frozen at -80°C in OCT medium, imaged for liposome, melanoma exosome and melanoma cell fluorescence using a Xenogen in vivo imaging system and cryosectioned. Central frozen tissue cross sections (8 μm thick) were fixed in acetone, stained for nuclei using VECTASHIELD mounting medium with DAPI (Vector Laboratories Inc., Burlingame CA) and visualized using fluorescent microsocopy to detect fluorescent carbocyanine labeled exosomes or cells within nodes or stained with eosin-haematoxylin to verify the structure of the lymphoid tissue.

**Real time reverse transcriptase (RT-RT) pcr analysis of lymph nodes.** Dissected lymph nodes were solubilized using Qiagen Qiazol solution and total RNA isolated using the Qiagen miRNeasy Mini kit (Qiagen Inc., Valencia, CA) according to the handbook protocol. For each node, 1 μg of total RNA was converted to cDNA using the RT2 first strand kit (C-03, SABiosciences, Frederick, MD) and analyzed on the SABiosciences PAMM-024 mouse angiogenesis array with an Applied Biosciences 7300 real time pcr machine. Even though the same amount of cDNA was applied to each array for either liposome or exosome treated nodes, these experimental conditions have not been assessed before and thus we further sought to standardize the amount of biological material between arrays. Using the method described by Viraj P. Mane et al., we determined the best normalization gene (tissue inhibitor of
melloproteinase 2) across 6 initial arrays (3 liposome and 3 exosome nodes) for our experimental conditions based on the criteria of minimal Ct variance, as reflected by lowest standard deviation, and highest normality of distribution (22). Normality of distribution was determined using JMP Version 8 (SAS Institute, Cary, NC) statistical software.

**Statistics.** To determine the statistical significance between the fluorescent distributions of individual lymph nodes, the 2-tailed Student’s t-test was used to calculate p values for $\alpha = 0.05$. For comparing overall lymph node (L PO, R PO, L IN, R IN) distribution patterns between liposomes, exosomes, melanoma cells chasing liposomes and melanoma cells chasing exosomes groups, JMP Version 8 (SAS Institute, Cary, NC) statistical software was utilized according to product instructions (http://www.jmp.com/support/notes/30/584.html) for replicate data (n = 5) using the univariate approach (F ratio = 5.94, $p < 0.0001$) so as not to exclude nodes containing a replicate measurement(s) where % fluorescent signal distribution = 0. Thus, a standard least squares matrix table was constructed and least squares means differences student's t analysis performed on all possible pairings between lymph node subtypes (exosome L PO vs. liposome L PO or liposome L PO vs. cells chasing liposomes L PO etc.) assuming a random normal distribution and $\alpha = 0.05$. For RT-RT pcr array analysis, RT2 Profiler PCR Array Software (SABiosciences, Frederick, MD) incorporating a 2-tailed Student’s t-test was used to calculate p values for $\alpha = 0.05$ (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

**RESULTS**

**Melanoma exosomes home to sentinel lymph nodes**

Given the predilection of melanoma to metastasize via lymphatics, we hypothesized that melanoma exosomes travel to sentinel lymph nodes. To the best of our knowledge this is the first study of its kind to assess lymphatic trafficking of any type of exosome. Therefore, we
constructed a control liposome to determine whether trafficking patterns would differ between melanoma exosomes versus inert bland nanovesicles lacking protein, mRNA, miRNA or other complex molecular variables found in exosomes (23-25). Based on our experience formulating stable nanoparticles, we constructed liposomes containing phosphatidyl choline (64.89 mol %), phosphatidyl ethanolamine (3.02 mol %), cholesterol (32.08 mol %) and a negligible amount of DiD (0.01 mol %) fluorescent label. These lipids are common components of exosomes (26-28). The use of these lipid ratios produced liposomes with a size (98 +/- 4 nm) and electrokinetic (zeta) potential (-8 +/- 2 mV) that closely approximate that of B16-F10 melanoma exosomes (95 +/- 14 nm and -11 +/- 5 mV). Thus, DiR labeled B16-F10 melanoma exosomes or an equivalent number of control DiD labeled bland liposomes were injected into the foot pads of albino C57BL/6 mice as described (21). Mouse feet are drained by a corresponding right and left pair of popliteal (PO) and inguinal (IN) lymph nodes (21). Thus, these nodes serve as sentinel nodes for footpad tumors. The IN nodes were chosen for extraction and analysis in this experiment rather than the PO nodes because we anticipated the potential for increased cross-trafficking between the more distal left and right IN lymphatics than the local sentinel PO nodes. Moreover, we sought to determine whether melanoma exosomes are capable of traveling long distances from the injection site. These sentinel nodes were obtained 48 hours after injection of liposomes or melanoma exosomes into the right or left foot pad respectively (Fig. 1A). Inguinal nodes were collected for each mouse ipsilateral or contralateral to the foot pad injection site of liposomes or exosomes and scanned for fluorescent signals using IVIS (Fig. 1B). The results demonstrate a significant homing preference of melanoma exosomes to the IN node ipsilateral to the injection site (Fig. 1C). In contrast, the liposome signal was distributed evenly in IN nodes both ipsilateral and contralateral to the injection site. Additionally, there was no difference in the average size of lymph nodes (Fig. 1D) or number of nucleated cells (Fig. 1E) present in either the ipsilateral or contralateral lymph nodes 48 hours following foot pad inoculation with either liposomes or melanoma exosomes. Taken together, these data
demonstrate a prominent and focal selectivity of melanoma exosome homing to the ipsilateral "sentinel" lymph nodes when compared to liposomes of similar size.

**Melanoma exosomes influence the lymph node distribution pattern of free melanoma cells.**

Based on the short term homing pattern of melanoma exosomes relative to liposomes (Fig. 1), we hypothesized that melanoma exosomes may influence how free melanoma cells distribute within a lymphatic microenvironment during metastasis. To approximate a metastatic process *in vivo*, we serially injected the left foot pad of two groups of mice three times, once every 48 hours, with either liposomes or melanoma exosomes. For the third injection, we included one million fluorescent green (carbocyanine, DiO) labeled melanoma cells. Foot pad tumors were visually apparent at 10 days in both groups. The average mass of the tumor sections obtained for the liposome and exosome mice groups was similar (33 and 34 milligrams respectively).

Ipsilateral and contralateral inguinal (IN) and popliteal (PO) lymph nodes were harvested from both groups of animals to assess the lymphatic distribution pattern of melanoma cells following prior inoculation with liposomes or exosomes. We chose the L PO and L IN nodes because they are known to drain the left foot pad injection site. R PO and R IN nodes were chosen given the potential for bilateral lymphatic cross trafficking of nanovesicles demonstrated previously (Fig. 1C). Fluorescent signals for melanoma cells, exosomes or liposomes were quantified for each node using IVIS. No difference in the lymphoid mass among any of the nodes was found with the exception of the ipsilateral L PO node, where the exosome treated group resulted in larger nodes (Fig. 2A). To normalize against any differences in particle or cell loading and fluorescence signal emission efficiencies between different dyes, all fluorescence signals for liposomes, exosomes, cells chasing liposomes and cells chasing exosomes were converted to % distribution for each mouse and averaged. This revealed a difference between
the distribution of exosomes and liposomes in the L PO and R IN nodes (Fig. 2B). Additionally, we converted the fluorescent signal for melanoma cells to an approximate cellular number using a standard curve relating fluorescence efficiency to cell number. This approach revealed no difference in the total cellular signal in the four nodes for each mouse between the liposome or exosome inoculated groups (Fig. 2C). We next compared the inter- and intra-nodal distribution pattern between particle and melanoma cellular groups using least squares matrix analysis (F ratio = 5.94, p < 0.0001). This revealed a significant difference between the melanoma cellular distribution pattern in the L PO nodes of animals pretreated with melanoma exosomes (Fig. 2D) that could be correlated to the difference in distribution patterns observed for liposomes and exosomes (Fig. 2B). These findings were further supported by fluorescent microscopy comparison of L PO nodes showing an increase in the number of melanoma cells infiltrating the larger node for the exosome versus liposome inoculation groups. This increase was preferentially located in the periphery of the node (Fig. 2E).

**Melanoma exosomes enhance migration of melanoma cells to melanoma exosome rich sites in sentinel lymph nodes.**

Based on the results of the melanoma cell recruitment experiments, we hypothesized that melanoma exosomes home to melanoma exosome sites in sentinel lymph nodes and were thus responsible for the difference in the pattern of distribution between melanoma cells chasing liposomes compared to melanoma cells chasing exosomes in the L PO node. To further investigate this hypothesis, additional longer term experiments (10 days) were performed as described in the previous section. Analysis of inter-group comparisons between liposomes and cells chasing liposomes groups revealed no difference (Fig. 3A, i). Intra- group comparisons within the liposome group demonstrated a difference in distribution between the R PO and R IN nodes (Fig. 3A, ii). The R PO versus R IN difference was also confirmed within the cells
chasing liposomes group (Fig. 3A, iii). However, in contrast to the liposomes group (Fig. 3A, ii), the cells chasing liposomes group (Fig. 3A, iii) also contained differences between the L PO and L IN nodes and the L IN and R PO nodes. Combined, the results of inter- and intra- group comparisons revealed both similarities and differences between the nodal distribution of liposomes versus cells chasing liposomes groups. For example, more liposomes (Fig. 3A, ii) than cells chasing liposomes (Fig. 3A, iii) distributed to the L PO node. In contrast, more cells chasing liposomes (Fig. 3A, iii) than liposomes (Fig. 3A, ii) distributed to the L IN node.

Analysis of inter-group comparisons between exosomes and cells chasing exosomes revealed no difference in the distribution pattern with the exception of the L PO node (Fig. 3B, i). Fluorescent microscopy analysis confirmed this finding demonstrating more exosome signal in the L PO node cross section than melanoma cell signal (Fig. 3C). Analysis of intra- group comparisons of the exosomes (Fig. 3B, ii) and cells chasing exosomes groups (Fig. 3B, iii) revealed the same differences between the L PO node and the L IN, R PO and R IN nodes. Similar to the 48 hour experiment (Fig. 1C), the majority of exosome (Fig. 3B, ii) versus liposome (Fig. 3A, ii) signal localizes ipsilateral to the left foot pad injection site.

These data demonstrate that melanoma cells assume a random lymphatic distribution pattern similar to but not identical to that of inert bland liposomes when chasing liposomes. However, if melanoma cells chase melanoma exosomes, the melanoma cells assume the same pattern of distribution as the exosomes where the majority of signal for both cells and exosomes is found in the significantly larger L PO node (Fig. 2B, i) closest to the left foot pad injection site.

**Melanoma exosome dependent lymph node metastasis is driven by induction of multiple metastatic pathways.**

Taken together, the results of our previous experiments demonstrate melanoma exosome dependent recruitment of melanoma cells to exosome rich sites in sentinel lymph nodes. Based on these results, we hypothesized that the mechanism of melanoma exosome
dependent lymph node metastasis is induction of metastatic pathways conducive to the trapping and growth of melanoma cells. We expected induction in the larger PO sentinel nodes, but opted to determine whether a single dose of melanoma exosomes could travel to and induce metastatic pathways long range in the more distal and less obvious IN sentinel nodes. We reasoned that this would more accurately demonstrate the potency of exosome influences on distal lymph node microenvironments as would be encountered with widespread metastasis. For these experiments, we normalized the gene expression in the R IN node (liposomal) and used it as the baseline for comparison to melanoma exosome induced gene expression in the L IN node. Thus, we compared left and right IN nodes for differential gene expression in mice at 48 hours post a single dose of exosome (left) and liposome (right) foot pad injections in individual mice. Of 84 paired genes assessed using an RT-RT pcr array, we discovered 13 significant differences (p < 0.05) (Fig 4). To simplify interpretation, we subdivided the identified genes into three groups: cell recruitment (Fig. 4A), extracellular matrix (Fig. 4B) and vascular growth factors (Fig. 4C). Overall, the array results demonstrate that melanoma exosomes enable diverse modes of gene induction within sentinel lymph nodes associated with traditional angiogenic pathways to the establishment of matrix architecture conducive to tumor recruitment and growth.

DISCUSSION

Metastatic progression is a complicated interplay between signaling molecules, tumor cells and immune cells that will likely differ between different tumor cell types that may or may not produce exosomes. Normal immune and non-immune exosomes are also likely to be involved in this process. Future experiments will be required to tease apart the complex interchange between normal and tumor exosomes at all stages of metastasis. To the best of our knowledge, our findings are the first to directly demonstrate native melanoma exosome induced lymph node conditioning in vivo. These findings are limited to exosomes as contrasted
to microparticles based on their characteristic features of differential density, size and morphology (15), (19).

Herein we present a novel tumor exosome dependent model of lymphatic metastatic progression that supports the hypothesis that preconditioned regional or sentinel lymph nodes play an active role in the progression of metastasis (5). We demonstrate that melanoma exosomes home to sentinel lymph nodes in vivo (Fig. 1). Further, we demonstrate that melanoma exosomes can recruit melanoma cells to sentinel lymph nodes (Fig. 2, 3). Finally, in the absence of melanoma cells, the mechanism of action responsible for this process is melanoma exosome dependent induction of metastatic factors (Fig. 4).

Thus metastatic factors responsible for the recruitment of melanoma cells to sentinel nodes are upregulated by melanoma exosomes themselves. Stabilin 1 (MS-1) expression on vasculature (29) is correlated to melanoma metastasis while upregulation of ephrin receptor β4 promotes migration and proliferation of melanoma cells (30-31). Additionally, melanoma cells derived from lymphatic metastasis express integrin αvβ3 which allows their recruitment to lymph nodes through interactions with vitronectin (32).

Our data further demonstrate that induction of sentinel nodes by melanoma exosomes increase the expression of a network of interconnected extracellular matrix factors that may promote trapping of melanoma cells within sentinel node niches. MAPK 14 (p38) (33), urokinase plasminogen activator (uPA) protease (34), collagen 18 (35) and laminin 5 (36) derivatives can remodel node stroma to permit basement membrane invasion by tumor cells, while G-α13 signaling is required for vascular organization during these processes (37).

Finally, the presence of melanoma exosomes in lymph nodes leads to induction of angiogenic growth factors necessary for melanoma growth. VEGF-B expression is increased by metastatic melanoma cells (38) and maintains survival of neovasculature (39). Increased HIF1-α expression by melanoma cells contributes to malignancy (40), increased VEGF expression (41) and poor prognosis (42). Paradoxically, thrombospondin 1 (Thbs1) can act on normal...
peripheral vasculature to increase melanoma blood flow at the expense of peripheral flow (43) and may therefore promote increased sentinel node blood flow conducive for tumor growth.

Tumor microenvironment associated TNF-α promotes melanoma growth and angiogenesis (44). Further, TNF-α is upregulated by myeloid derived suppressor cells (MDSCs) induced by melanoma microvesicles and granulocyte-macrophage colony stimulating factor (GM-CSF) (45). Induction of TNF-α therefore signifies simultaneous upregulation of angiogenic and immunosuppressive activities by native melanoma exosomes in the lymph node microenvironment. This supports our previous work demonstrating simultaneous induction of angiogenesis and immunosuppressive factors (GM-CSF and TNF-α) by melanoma exosomes (15) and is consistent with other in vitro reports demonstrating melanoma microvesicle mediated “counterattack” of anti-tumor T-cells (8) and induction of MDSCs (11),(46) which suppress anti-tumor T-cell function (45).

Collectively, increased gene expression of cell recruitment, extracellular matrix and vascular proliferation factors by melanoma exosomes produces a niche within sentinel node microenvironments conducive to melanoma cell recruitment, trapping and growth. Essentially, melanoma exosomes serve as the “seed” and sentinel nodes the “soil” for melanoma metastasis. This “turf preparation” response is further supported by our data demonstrating that the pattern of tumor cell recruitment is not random, in contrast to that observed for cells chasing inert liposomes. Rather, trafficking after exosomes is preferential for localization to sentinel nodes closest to footpad tumors. This is exemplified by the recruitment of the majority of melanoma cells to the left popliteal sentinel node where they are buffered by an even greater number of melanoma exosomes. Given our data, and the numerous reports of tumor exosome mediated immune suppression (11-13) it seems logical that tumor exosomes would have a role in conditioning sentinel lymph nodes for the controlled spread of metastasis whereby routes of communication between primary and metastatic tumors can be efficiently maintained.
The complexity of the mechanism of action of melanoma exosomes on sentinel nodes implies additional testable hypotheses. Chief among them is whether the component parts of melanoma exosomes coordinately or independently signal nodal preparation: i.e., what is the role of surface molecular epitopes derived from the melanoma parent cell versus their contained cargos. Essentially, exosomes carry and protect fragile mRNA, miRNA and proteins within their core (23-25). Without this protective environment and molecular targeting by a lipid shell, free mRNA or miRNA would otherwise be rapidly degraded (47) and exosome contents rendered undeliverable and ineffective. Furthermore, the exosome shell necessarily expresses a specific configuration of targeting motifs required for their interaction and communication with target cells.

In our previous report, melanoma exosomes were observed to influence endothelial tubule morphology and stimulate the production of endothelial spheroids and sprouts in a dose-dependent manner (15). In concert, tumor exosomes simultaneously elicited paracrine endothelial signaling by regulation of certain inflammatory cytokines. Taken together, these findings demonstrate that melanoma exosomes are capable of directly tuning a remote lymph node toward a microenvironment that facilitates melanoma growth and metastasis in lymph nodes even in the local absence of tumor cells (Fig. 5). Thus melanomas and perhaps other tumors can take advantage of an efficient exosomal messenger mechanism to signal site preparation for eventual metastasis that is accomplished through pre-metastatic conditioning of lymph nodes by a vanguard of tumor exosomes.
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J. L. Hood and S. A. Wickline devised experiments, wrote and edited the manuscript. J. L. Hood is the principle investigator of the exosome project and performed and analyzed the experiments. Susanna S. Roman developed procedures for the isolation, processing, and histological analysis of lymph nodes. All authors read and approved the final version of the manuscript. Additionally, we would like to thank Stacy J. Allen who provided expertise in animal care and Michael J. Scott who cultured cells, isolated exosomes and assisted with injections.

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FIGURE LEGENDS

Figure 1. Melanoma exosome versus control liposome inguinal lymph node distribution in vivo at 48 hours. A, Albino C57/BL6 mice were anesthetized under 2.5% isofluorane and foot pads injected with 2.5% Evans Blue dye in 25 μl PBS to visualize lymph nodes (21). At 20 min. post injection mice were euthanized, dissected and right (R) and left (L) inguinal (IN) nodes visualized (red circles). B, DiD-liposome versus DiR-exosome signal detected by IVIS in representative pairs of IN nodes (n = 6) both ipsilateral and contralateral to the foot pad injection site. C, DiD-liposome versus DiR-exosome average signal distribution in IN nodes (n = 6 pairs). Error bars represent s.e.m. D, Following dissection, inguinal lymph nodes were weighed for comparison between liposome and exosome treated nodes (n =5 pairs); error bars represent s.d. E, Using H&E staining, the number of nucleated cells in DiD-liposome or DiR-exosome treated nodes were counted and averaged for (n = 15) random fields obtained from 3 nodes for each treatment with 5 fields per node; error bars represent s.d. Statistically significant relationships are delineated by connecting bars. Rounded p-values are listed above the connecting bars; p values < 0.05 were considered statistically significant.

Figure 2. Lymph node distribution of melanoma cells in liposome versus exosome treatment groups post 10 days tumor challenge. A, average lymph node mass for four liposome versus melanoma exosome treated lymph nodes (L PO, L IN, R PO, R IN). Error bars represent s.d. (n = 5 mice). B, distribution pattern of liposomes and melanoma exosomes. Error bars represent s.e.m. (n = 5 mice). C, average number of melanoma cells detected for all four liposome or exosome treated nodes. Error bars represent s.d. (n = 5 mice). D, distribution pattern of melanoma cells in liposome or exosome treatment groups. Error bars represent s.e.m. (n = 5 mice). E, fluorescent microscopy of central L PO lymph node cross sections in cells chasing liposomes versus exosomes treatment groups; representative of (n = 5) mice. Any statistically
significant relationships are delineated by connecting bars. Rounded p-values are listed above the connecting bars; p values < 0.05 were considered statistically significant. L = left, R = right, PO = popliteal, IN = inguinal. Nuclei of lymph node cells were stained with DAPI (blue). Melanoma cells were fluorescently stained with DiO (green) before injection.

**Figure 3.** Lymph node distribution of melanoma versus liposome treatment groups post 10 days tumor challenge. **A**, distribution pattern of liposomes and cells chasing liposomes. Inter-group distribution (\(i\)), liposome intra-group distribution (\(ii\)), cells chasing liposomes intra-group distribution (\(iii\)). **B**, distribution pattern of exosomes and melanoma cells chasing exosomes. Inter-group distribution (\(i\)), exosome intra-group distribution (\(ii\)), cells chasing exosomes intra-group distribution (\(iii\)). **C**, fluorescent microscopy of a central left popliteal lymph node cross section in the exosome treatment group. Error bars represent s.e.m. for (n = 5 mice). Any statistically significant relationships are delineated by connecting bars. Rounded p-values are listed above the connecting bars; p values < 0.05 were considered statistically significant. Nuclei of lymph node cells were stained with DAPI (blue). Melanoma cells were fluorescently stained with DiO (green) and melanoma exosomes with Dil (red) before injection.

**Figure 4.** RT-RT pcr analysis of Exosome versus Control Liposome Induced Gene Expression in Inguinal Lymph Nodes at 48 hours. Bars represent average fold changes for induction of sentinel node factors related to **A**, melanoma cell recruitment to sentinel nodes, **B**, matrix modifiers promoting trapping of melanoma cells within sentinel nodes, and **C**, angiogenic growth factors promoting melanoma growth in sentinel nodes. Control (liposome) fold changes are all normalized to 1 (cross bar). Error bars represent the s.e.m. for (n = 6 ) arrays. Rounded p-values are listed above the error bars for each gene; p values < 0.05 were considered statistically significant. The abbreviations R = receptor, Mapk = map kinase, uPA = urokinase plasminogen activator, Col. = collagen, Lam. = laminin, ip2 = inducible protein 2, TNF = tumor.
necrosis factor, VEGF = vascular endothelial growth factor, HIF = hypoxia inducible factor and Thbs = thrombospondin.

**Figure. 5.** Preparation of sentinel lymph nodes for tumor metastasis by melanoma exosomes. 
A, Melanoma exosomes home to sentinel lymph nodes.  
B, Within sentinel nodes, melanoma exosomes prepare a pre-metastatic niche by inducing expression of factors responsible for cell recruitment, matrix remodeling and angiogenesis (15) and likely mediate immunosuppression (12),(45).  
C, Metastatic melanoma or stem cells travel to the prepared niche where they encounter a microenvironment conducive to tumor cell adherence and growth.  
MDSC = myeloid derived suppressor cell.
Figure 2

A

Lymph node mass (mg)

Liposomes Exosomes

p = 0.003

L PO LIN R PO R IN

B

% Distribution

p < 0.0001

p = 0.02

L PO LIN R PO R IN

C

Melanoma Cells

Chasing Liposomes Chasing Exosomes

(40 X)

D

% Distribution

p = 0.001

L PO LIN R PO R IN

E

Melanoma Cells

Nuclei

Chasing Liposomes Chasing Exosomes

(40 X)

Merge
Figure 4

A

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B

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Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis

Joshua L Hood, Susana San Roman and Samuel A Wickline

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